PAGE

1 AGENCY USE ONLY	2 REPORT DATE	3 TYPE/DATES COVERED
	1993	

4 TITLE AND SUBTITLE PROTECTION BY TACRINE AND SOME ADJUNCTS AGAINST THE DEPRESSANT EFFECTS OF SOMAN IN GUINEA-PIG ATRIUM

5 FUNDING NUMBERS

6 AUTHOR WAI-MAN LAU

7 FORMING ORG NAMES/ADDRESSES DEFENCE SCIENCE AND TECHNOLOGY ORGANIZATION, MATERIALS RESEARCH LABORATORY, PO BOX 50, ASCOT VALE VICTORIA 3032 AUSTRALIA

8 PERFORMING ORG. REPORT NO

09 SPONSORING/MONITORING AGENCY NAMES AND ADDRESSES

11 SUPPLEMENTARY NOTES

12 DISTRIBUTION/AVAILABILITY STATEMENT

DISTRIBUTION STATEMENT A

12B DISTRIBUTION CODE

13. ABSTRACT (MAX 200 WORDS): THE NEGATIVE INOTROPIC EFFECTS OF SOMAN HAVE BEEN REPORTED PREVIOUSLY. IT WAS SUGGESTED THAT THE DEPRESSION IN ATRIAL FORCE OF CONTRACTION WAS A CONSEQUENCE OF CONTINUOUS MUSCARINIC RECEPTOR ACTIVATION BY EXCESSIVE ACETYLCHOLINE (ACh) ACCUMULATION AND ALSO POSSIBLY THROUGH DIRECT INTERACTIONS AT THE RECEPTOR-ASSOCIATED K+ CHANNELS BY ORGANOPHOSPHATE (OP). IN THIS STUDY, THE PROTECTIVE EFFECTS OF TACRINE (THA), AN ANTIMUSCARINIC AS WELL AS A K+ CHANNEL BLOCKER, AGAINST SOMAN IN GUINEA-PIG ATRIUM WERE INVESTIGATED. IT WAS FOUND THAT TACRINE COULD ANTAGONIZE THE NEGATIVE INOTROPIC EFFECTS OF SOMAN, THIS ANTAGONISM OCCURRED IN A CONCENTRATION DEPENDENT MANNER, WITH EFFECTIVE CONCENTRATIONS (ECs) FOR TACRINE RANGING FROM 1.7 TO 12.1 uM WHEN THE ATRIUM WAS EQUILIBRATED WITH 0.05-10 u M SOMAN. INCLUSION OF AN OXIME HI-6 (100 uM) IN THE REGIMEN IMPROVED THE EFFECACY OF TACRINE AGAINST SOMAN (LuM) BY 16.1 FOLD. ADDITION OF A POTENT ANTIMUSCARINIC, EITHER ATROPINE OR GLYCOPYRROLATE WITH TACRINE ALSO IMPROVED TACRINE'S EFFICACY AGAINST SOME SIGNIFICANTLY. ATROPINE, AT EQUIVALENT CONCENTRATION, APPEARED TO BE THE MOST EFFECTIVE OF THE THREE. AT 0.1 uM CONCENTRATION, ATROPINE WAS 4.25 AND 3.47 TIMES MORE POTENT THAN HI-6 AND GYLCOPYRROLATE RESPECTIVELY IN ENHANCING THA EFFICACY. OUR RESULTS SUGGESTED THAT THE IMMEDIATE SUPPRESSION OF THE MUSCARINIC MANIFESTATIONS AND THE REACTIVATION OF THE ENZYME ACETYLCHOLINESTERASE FOR THE REMOVAL OF EXCESS ACH ARE BOTH CRITICAL IN MAINTAINING THE MECHAMICAL FUNCTIONS OF A HEART DURING ACUTE OP POISONING THE BLOCKADE OF K+ CHANNELS BY TACRINE MAY ALSO CONTRIBUTE TO COUNTERING THE DEPRESSANT EFFECTS OF SOMA.N.

14 SUBJECT TERMS

15 NUMBER OF PAGES

7

16 PRICE CODE

17 SECURITY CLASS.REPORT	18 SEC CLASS PAGE	19 SEC CLASS ABST.	20 LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASS	

Gen. Pharmac. Vol. 24, No. 6, pp. 1513–1519, 1993
Printed in Great Britain

0306-3623/93 $6.00 + 0.00
Pergamon Press Ltd

PROTECTION BY TACRINE AND SOME ADJUNCTS AGAINST THE DEPRESSANT EFFECTS OF SOMAN IN GUINEA-PIG ATRIUM

WAI-MAN LAU*

Materials Research Laboratory, Defence Science and Technology Organization, P.O. Box 50, Ascot Vale, Victoria 3032, Australia [*Fax* (03) 246-8999]

(Received 16 February 1993)

Abstract—1. The negative inotropic effects of soman have been reported previously. It was suggested that the depression in atrial force of contraction was a consequence of continuous muscarinic receptor activation by excessive acetylcholine (ACh) accumulation and also possibly through direct interactions at the receptor-associated K^+ channels by organophosphate (OP).

2. In this study, the protective effects of tacrine (THA), an antimuscarinic as well as a K^+ channel blocker, against soman in guinea-pig atrium were investigated.

3. It was found that tacrine could antagonize the negative inotropic effects of soman. This antagonism occurred in a concentration dependent manner, with effective concentrations (ECs) for tacrine ranging from 1.7 to 12.1 μM when the atrium was equilibrated with 0.05–10 μM soman.

4. Inclusion of an oxime HI-6 (100 μM) in the regimen improved the efficacy of tacrine against soman (1 μM) by 16.1 fold.

5. Addition of a potent antimuscarinic, either atropine or glycopyrrolate with tacrine also improved tacrine's efficacy against soman significantly.

6. Atropine, at equivalent concentration, appeared to be the most effective of the three. At 0.1 μM concentration, atropine was 4.25 and 3.47 times more potent than HI-6 and glycopyrrolate respectively in enhancing THA efficacy.

7. Our results suggested that the immediate suppression of the muscarinic manifestations and the reactivation of the enzyme acetylcholinesterase for the removal of excess ACh are both critical in maintaining the mechanical functions of a heart during acute OP poisoning. The blockade of K^+ channels by tacrine may also contribute to countering the depressant effects of soman.

INTRODUCTION

Tacrine is a tricyclic primary amine. Its pharmacological properties have recently been reviewed (Freeman and Dawson, 1991). Tacrine has been shown to have moderate antimuscarinic activities (Drukarch *et al.*, 1988). Selective radioligands such as pirenzepine and *N*-methylscopolamine were displaced from the muscarinic M_1 and M_2 receptors respectively by tacrine with K_i values in the micromolar range (Freeman *et al.*, 1988; Kunysz *et al.*, 1988). Tacrine also inhibited the binding of specific radioligands at a number of receptor types including adrenergic α_1, α_2 and β, dopamine D_1 and D_2; serotonin S_1 and S_2; NMDA; adenosine A_1 and PCP receptors although the concentrations of tacrine required to inhibit 50% of radioligand binding were relatively high (Albin *et al.*, 1988; Drukarch *et al.*, 1988; Zhu *et al.*, 1988; Nielsen *et al.*, 1989). It was further shown that tacrine antagonized the shortening of action potential in guinea-pig atrium by carbachol and adenosine; and increased the force of atrial contraction (Freeman *et al.*, 1988). This was apparently linked to its interactions at the ionic channels, as was evidenced by the blocking actions of tacrine on the inwardly rectifying K^+ current in guinea-pig isolated ventricular myocytes (Osterrieder, 1987) and the slow outward K^+ current in the peptidergic neurons of snail (Drukarch *et al.*, 1987). Tacrine was found to inhibit cholinesterases (Shaw and Bentley, 1953). This inhibition is probably reversible and mixed, with higher potency on butyrylcholinesterase than acetylcholinesterase (Dawson, 1990; Dawson *et al.*, 1991).

Our previous studies indicated that the negative inotropic effects of soman, a potent irreversible anticholinesterase in guinea-pig atrium could be reversed by atropine, edrophonium, amantadine and 4-aminopyridine (Lau and Freeman, 1989). The antagonism, however, could not be explained solely on the basis of excessive acetylcholine accumulation. The findings that some K^+ channel blockers were effective in reversing the depressant effects of soman suggested that direct interaction at the K^+ channels by soman is possible. However the concentrations of the blockers required for complete reversal were in the milli-

*All correspondence should be addressed to Dr Tony Lau at the address shown.

molar range. To minimize undesirable side-effects, a more potent drug should be used to substitute these K^+ channel blockers. Tacrine, with its diverse pharmacological properties, appears to be therapeutically suitable for the treatment of organophosphate poisoning.

In this study, the protective effects of THA against the depressant effects of soman in guinea-pig atrium were investigated. In addition, potent antimuscarinics such as atropine and glycopyrrolate; and the AChE reactivator, HI-6 were included in the regimen for comparison of their potency to improve the efficacy of THA.

MATERIALS AND METHODS

Guinea-pig atrial preparations

Left atrial preparations were surgically removed from guinea-pigs of both sexes weighing 250–400 g as described by Freeman and Turner (1974). Preparations were transferred to an organ bath containing Ringers heart solution (composition in mM: NaCl 115, KCl 4.6, $CaCl_2$ 1.8, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 22, glucose 22, pH = 7.4) at 37°C. The buffer was continuously aerated with carbogen (95% O_2 and 5% CO_2). The preparations were allowed to equilibrate for 30 min or until they settled before the experiment commenced. The atria were stimulated at 2 Hz and 10 V and the tension of isometric contraction was recorded by a Shinkoh U-gauge (UL-2-120). The signal outputs were modulated by a Coulbourne transducer (S72-75) and recorded by a Graphtech linear recorder (WR-3071). Tension development was constant during the period of the experiments which lasted for approx. 3 hr.

Drug administration

The concentration–response curves were constructed by adding drugs cumulatively to the organ bath. The adjuncts except for HI-6 were mixed with soman before administering to the atrial preparations. In the case of HI-6, soman was introduced first; HI-6 was added after the preparations had achieved a steady contraction force. This would allow soman to exert its effects on the preparations or otherwise be completely hydrolysed by HI-6. Data are presented as mean ± SEM.

Chemicals

Soman was synthesized in house with a purity greater than 99%. HI-6 was a gift from the Defence Research Establishment Suffield, Alberta, Canada (batch DRES-32); glycopyrrolate was donated by Robbins Pty Ltd, Sydney. Tacrine was obtained from the Institute of Drug Technology, Melbourne and atropine was purchased from Sigma, St Louis, U.S.A. All other chemicals used were A.R. grade and readily available from commercial sources.

RESULTS

Effects of tacrine on the negative inotropic effects of soman

Soman reduced the force of atrial contraction in a concentration-dependent manner. A 6.6% reduction was observed at 0.05 μM soman and a 58% decrease in contraction force was recorded at 10 μM (Table 1). These observations were in conformity with those previously reported (Lau and Freeman, 1989).

Table 1. Effects of soman on the atrial force of contraction. Data are expressed as mean ± SEM and were obtained from 6 experiments for each soman concentration

Soman (μM)	Baseline contraction force force (mg)	Contraction force after soman (mg)	Percent reduction
0.05	609 ± 126	569 ± 113	6.6
0.5	828 ± 134	552 ± 103	33.3
1.0	630 ± 75	356 ± 47	43.5
10.0	424 ± 24	117 ± 12	58.3

Tacrine could completely reverse the negative inotropic effects of soman. Such antagonism was concentration-dependent (Fig. 1). The effective concentrations of tacrine (ECs) which could return the atrial contraction force back to the control increased with increasing concentration of soman (Fig. 2). At 0.05 μM soman, the EC for THA was 1.73 μM and at 10 μM soman, 12.1 μM of THA was needed. Increasing THA above its EC against soman could result in further increases in atrial contractility. In the presence of 10 μM soman, THA (20 μM) would increase the contraction force by 3.24 times the basal value.

Combined effects of tacrine and atropine against soman

Atropine, in combination with tacrine was effective in antagonizing the depression of atrial contraction due to 1 μM soman (Fig. 3). Increasing the concentration of atropine from 0.01–0.1 μM caused a significant shift of the THA concentration–response curve to the left. A further increase in atropine concentration to 1 μM moved the THA concentration–response curve back to the right; suggesting a reduction in the efficacy of tacrine against soman. The ECs of tacrine against 1 μM soman in the presence of 0.01, 0.1 and 1 μM atropine were 3.20, 0.99 and 5.73 μM respectively (Table 2). This represented a 1.94 11.24 times enhancement in the efficacy of THA against soman.

Combined effects of tacrine and glycopyrrolate against soman

Glycopyrrolate is a potent antimuscarinic (Lau and Szilagyi, 1992). It could also improve the efficacy of THA against soman by shifting the THA concentration–response curve to the left, although this improvement in comparison to atropine was somewhat smaller (Fig. 4). At 0.01 μM glycopyrrolate, the EC of tacrine required to fully reverse the negative inotropic effects of 1 μM soman was 3.55 μM (Table 2); i.e. a 3.13-fold increase in the efficacy of THA. Increasing the concentration of glycopyrrolate to 0.1 and 1 μM progressively improved the efficacy of tacrine by 3.23 and 7.08

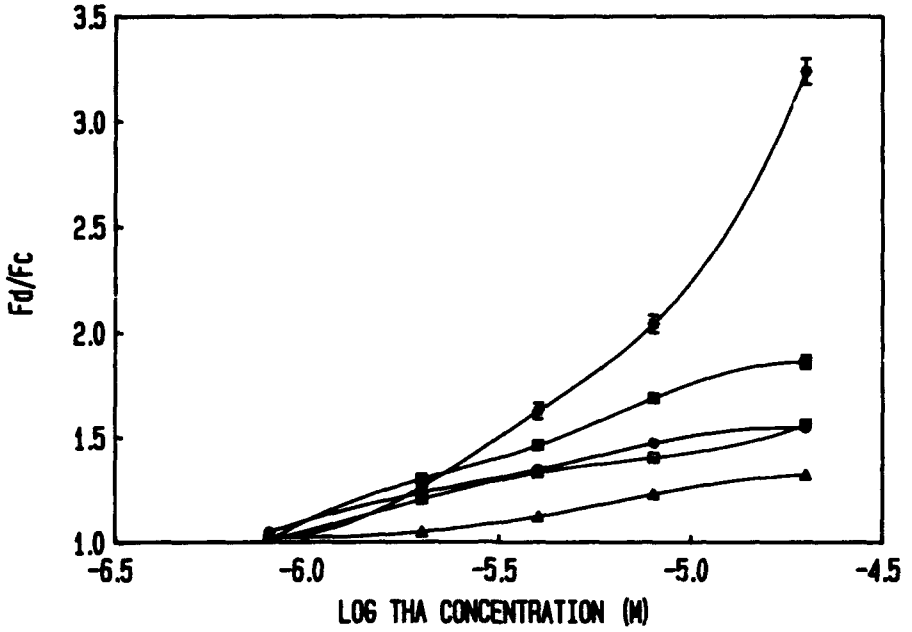

Fig. 1. Antagonism of the negative inotropic effect of soman by tacrine. ●: 0; ▲: 0.05 μM; □: 0.5 μM; ■: 1 μM; ✱: 10 μM soman. Fd/Fc (baseline) = Force in the presence of tacrine alone/Baseline force prior to the addition of tacrine. Fd/Fc (soman) = Force in the presence of soman and tacrine/Force in the presence of soman alone. Data are expressed as mean ± SEM, $n = 6$.

times, suggesting that the enhancement was concentration dependent.

Combined effects of tacrine and HI-6 against soman

The oxime HI-6 can itself antagonize the depressant effects of 1 μM soman (Table 3). This antagonism was only marginal at 0.1 and 1 μM HI-6, but further increases in concentrations resulted in a 14 and 28% increase in contraction force after soman. In combination with THA, HI-6 shifted the THA concentration–response relationship curves to the left. The shift was concentration dependent and was most

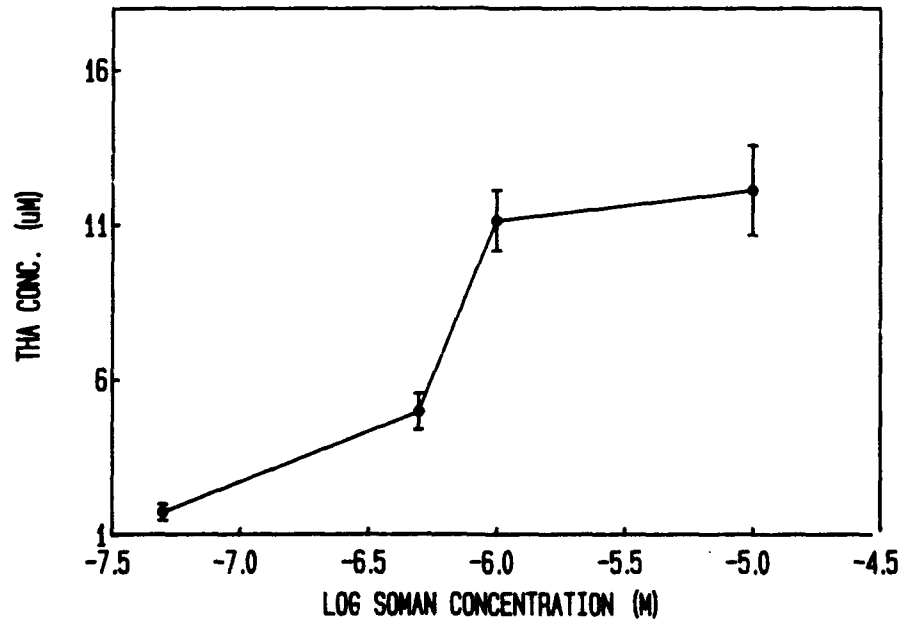

Fig 2. Effect of soman on the effective concentration of tacrine to restore atrial force of contraction to pre-soman levels. Data are expressed as mean ± SEM, $n = 6$.

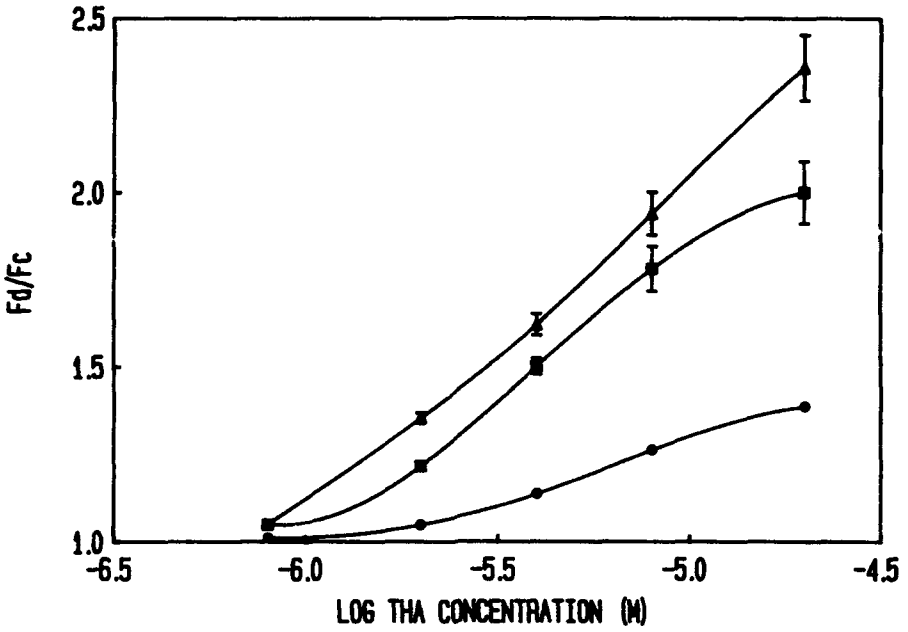

Fig. 3. Effect of atropine on the antagonism of the negative inotropic effect of soman by tacrine. ●: 0.01 μM atropine + 1 μM soman; ▲: 0.1 μM atropine + 1 μM soman; ■: 1 μM atropine + 1 μM soman. Fd/Fc = Force in the presence of soman, tacrine and adjunct/Force in the presence of soman alone. Definition of Fd/Fc for Fig. 3 also applies to Figs 4 and 5. Data are expressed as mean ± SEM, $n = 5$.

effective at 100 μM HI-6 (Fig. 5). This was reflected in the lowering of the ECs of tacrine required to reverse the depression in atrial contractility due to soman. Table 2 shows the values of the ECs which were 4.21, 3.62, 1.16 and 0.69 μM in the presence of 0.1, 1, 10 and 100 μM HI-6 respectively. This represented a 2.64–16.13-fold increase in the efficacy of THA against soman.

DISCUSSION

Tacrine protects the atrium against reductions in contraction force due to soman in a concentration-dependent manner. It was observed that this protection is fully effective even when soman concentrations tested (up to 10 μM) far exceeded those required (< 100 nM) for complete inhibition of AChE (Zhao *et al.*, 1983). This antagonism apparently cannot be linked to further accumulation of ACh by increasing soman concentration beyond 100 nM. Interactions between tacrine and soman at another site in close association with the cholinergic receptors are possible.

Table 2. Effective concentrations (EC) of tacrine to restore the atrial force of contraction to pre-soman levels after equilibrium with soman (1 μM) plus adjuncts of different concentrations. Data are expressed as mean ± SEM and were obtained from 5 experiments for each drug treatment

Drug treatment		EC for tacrine (μM)
Soman (1 μM)	plus atropine (0.01 μM)	3.20 ± 0.06
	plus atropine (0.1 μM)	0.99 ± 0.13
	plus atropine (1 μM)	5.73 ± 1.41
Soman (1 μM)	plus glycopyrrolate (0.01 μM)	3.55 ± 0.60
	plus glycopyrrolate (0.1 μM)	3.44 ± 1.23
	plus glycopyrrolate (1 μM)	1.57 ± 0.74
Soman (1 μM)	plus HI-6 (0.1 μM)	4.21 ± 1.23
	plus HI-6 (1 μM)	3.62 ± 0.49
	plus HI-6 (10 μM)	1.16 ± 0.27
	plus HI-6 (100 μM)	0.69 ± 0.20

It has been demonstrated that tacrine blocked K^+ channels in isolated ventricular myocytes of guinea-pig. Both the inward rectifying and the delayed outward K^+ currents were modified (Osterrieder, 1987). Drukarch *et al.* (1987) studied the effects of tacrine on K^+ conductance in peptidergic neurons of snail and suggested that the slow outward K^+ current was inhibited. Studies by Stevens and Cotman (1987), Rogawski (1987) and Reiner and McGeer (1988) also confirmed the K^+ channel blocking properties of tacrine. Our work on guinea-pig atrium also showed the blocking of receptor-associated K^+ channels by tacrine (Freeman *et al.*, 1988). The blockade of K^+ channel is believed to lead to an increase in the conductance of Ca^{2+} current which would subsequently cause an increase in the force of contraction (Freeman, 1979; Lau and Freeman, 1989).

The depressant effects of soman on guinea-pig atrium were suggested to comprise two components (Lau and Freeman, 1989). The first is a result of AChE inhibition, which results in excessive

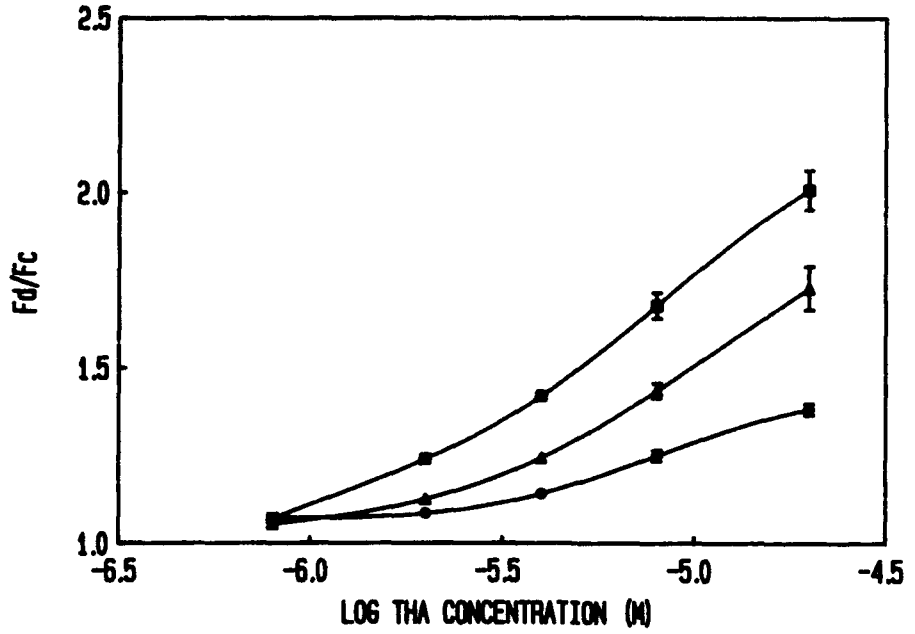

Fig. 4. Effect of glycopyrrolate on the antagonism of negative inotropic effect of soman by tacrine. ●: 0.01 μM glycopyrrolate + 1 μM soman; ▲: 0.1 μM glycopyrrolate + 1 μM soman; ■: 1 μM glycopyrrolate + 1 μM soman. Data are expressed as mean ± SEM, $n = 5$.

acetylcholine accumulation and cholinergic stimulations. The second is believed to be associated with K^+ channel activations.

Support for the second component can be found in studies by Pascuzzo *et al.* (1984) and Albuquerque *et al.* (1984, 1985) who reported interactions by some organophosphorus compounds at the ionic channels of the nicotinic-ACh receptors. Edrophonium, amantadine and 4-aminopyridine, either known or putative K^+ channel blockers were capable of countering the negative inotropic effects of soman (Lau and Freeman, 1989). These findings suggested that the antagonism between tacrine and soman on atrial contractility could be related to their interactions at the K^+ channels in atrium.

The inclusion of a potent antimuscarinic, atropine or glycopyrrolate to tacrine significantly improved its efficacy against soman in guinea-pig atrium. It was noted that the concentration–response curve of tacrine was shifted to the left when atropine was increased from 0.01 to 0.1 μM. A further increase to 1 μM reduced the efficacy of tacrine against soman (1 μM) by shifting the tacrine concentration–response curve back to the right, reducing the efficacy improvement from 11.24 to 1.94 times the control. Compared to atropine, glycopyrrolate was less effective in enhancing the efficacy of tacrine. Increasing the concentration of glycopyrrolate from 0.01 to 0.1 μM only marginally increased the efficacy of THA from 3.13- to 3.23-fold. At 1 μM glycopyrrolate, the improvement was 7.08-fold. The muscarinic antagonists prevent cholinergic manifestations basically by competing with ACh at the muscarinic receptor sites (Ellin, 1982). Previous studies indicated that 20 nM of atropine was sufficient to return contraction force to control level when atrium was equilibrated with 0.2 μM soman (Lau and Freeman, 1989). This was explained in terms of the high affinity of atropine with the muscarinic receptors. With an affinity constant of $2 \times 10^9\ M^{-1}$ (Birdsall and Hulme, 1978), atropine, at 20 nM or above, would occupy all muscarinic receptors. The reduction of tacrine's efficacy at higher concentrations of atropine may be an indication that a secondary interaction site is involved. Activation of this site presumably requires higher atropine concentrations and would produce opposing pharmacological effects generated by lesser atropine. The second muscarinic antagonist tested, glycopyrrolate has similar potency to atropine and has a preference in binding with the M_2 muscarinic subtypes (Lau and Szilagyi, 1992). The smaller

Table 3. Effect of HI-6 on the force of atrial contraction after soman (1 μM). Data are expressed as mean ± SEM and were obtained from 5 experiments from each drug treatment

HI-6 (μM)	Percent increase in force of contraction after soman (1 μM)
0.1	1.55 ± 2.53
1	4.63 ± 2.96
10	14.83 ± 3.65
100	27.96 ± 5.47

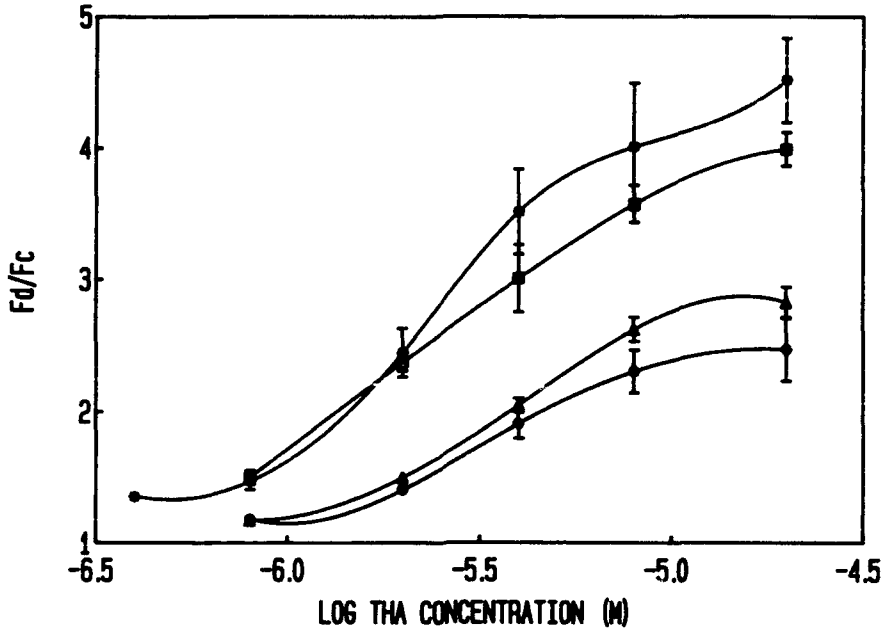

Fig. 5. Effect of HI-6 on the antagonism of the negative inotropic effect of soman by tacrine. ●: 0.1 μM HI-6 + 1 μM soman; ▲: 1 μM HI-6 + 1 μM soman; ■: 10 μM HI-6 + 1 μM soman; *: 100 μM HI-6 + 1 μM soman. Data are expressed as mean ± SEM, $n = 5$.

improvement in the efficacy of tacrine against soman in comparison to atropine was therefore unexpected. However, glycopyrrolate was reported to have multiple pharmacological properties. The one which could directly antagonize the positive inotropism of tacrine is the blockade of Ca^{2+} channels in the atrium (Lau and Szilagyi, 1992) although the concentration of glycopyrrolate administered in the present study was lower than that reported to block Ca^{2+} channels. This raises the possibility that a high concentration of atropine would also block Ca^{2+} channels which would explain the reverse in the efficacy of tacrine. Further studies are needed to clarify this point.

The oxime HI-6 was capable to antagonize the depressant effects of soman by itself. A 28% increase in the contraction by 100 μM HI-6 after soman (1 μM) was observed (Table 3). HI-6 in combination with tacrine was effective for antagonizing the depressant effects of soman. This effect was concentration dependent and was most prominent at 100 μM HI-6, the highest concentration tested (Table 3). The primary therapeutic value of HI-6 against soman is associated with its ability to reactivate the inhibited acetylcholinesterase (Clement *et al.*, 1991). The reactivated enzyme would hydrolyze the accumulated ACh, thus restoring the cholinergic neurotransmissions back to *status quo*. Combined with tacrine, the therapeutic effectiveness was further improved as tacrine has already spared some cholinesterases from irreversible inhibition due to soman and also competed with ACh at the receptor site. The mechanism for HI-6 alone to antagonize the depressant effects of soman is unknown at this stage. HI-6 was suggested to possess moderate *in vitro* and *in vivo* antimuscarinic activities (Valdes, 1985; Rousseaux and Dua, 1989). The actual site of interaction has not been identified but was believed to be allosterically linked to the muscarinic site (Kloog and Sokolovsky, 1985). Whether these mild antimuscarinic properties of HI-6 are responsible for the partial recovery of the atrial contractility after soman, remains to be determined.

In conclusion, it was shown that tacrine could protect guinea-pig atrium from the depressant effects of soman. The protection is a result of the multiple pharmacological activities of tacrine. These included the blockade of the muscarinic receptors and their associated K^+ channels, and the reversible inhibition of acetylcholinesterases which contribute to the prophylaxis against soman. When combined with more potent antimuscarinics and AChE reactivator, the efficacy of tacrine was futher enhanced. At an equivalent concentration (0.1 μM), atropine was 4.25 and 3.47 times more effective in enhancing tacrine against soman than HI-6 and glycopyrrolate respectively. The marked improvement in the protective effects of tacrine by these adjuncts suggested that the reactivation of AChEs and the blockade of muscarinic receptors are critical for maintaining normal heart functions in acute OP poisoning. The blockade of

K^+ channels by tacrine is also beneficial in countering the negative inotropic effects of soman.

Acknowledgements—The author wishes to thank Mr D. Amos for the preparation of soman and Mr M. Poretski for technical assistance.

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